MATERIALS AND METHODS RELATING TO NEURONAL DEVELOPMENT

Field of the Invention

The present invention relates to the induction of the neuronal
fate in neural stem cells or neural progenitor cells. It
relates to the induction of a specific neuronal phenotype, and
particularly to the induction of a midbrain dopaminergic
neuronal phenotype.

Background of the Invention

Neural stem cells have the ability to differentiate into
neurons, astrocytes and oligodendrocytes. Recent advances in
neural stem cell biology have shown that such stem cells can
be isolated, expanded, and used as source material for brain
transplants (Snyder, E. Y. et al. Cell 68, 33-51 (1992);
Rosenthal, A. Neuron 20, 169-172 (1998); Gage, F.H., et al.
Ann. Rev. Neurosci. 18, 159-192 (1995); Weiss, S. et al.
Trends Neurosci. 19, 387-393 (1996); Snyder, E. Y. et al.
Clin. Neurosci. 3, 310-316 (1996); Martínez-Serrano, A. et al.
Trends Neurosci. 20, 530-538 (1997); McKay, R. Science 276,
66-71 (1997); Studer, L. et al. Nature Neurosci. 1, 290-295
(1998)). However, although multiple studies demonstrate that
implanted neural stem cells successfully engraft and assume
legitimate neural phenotypes, when transplanted into the
intact adult brain these cells seem biased toward astro- and
oligodendroglial fates (Martínez-Serrano, A. et al. Trends
Neurosci. 20, 530-538 (1997); McKay, R. Science 276, 66-71
(1997); Snyder, E. Y. et al. Proc. Natl. Acad. Sci. USA 94,
11663-11668 (1997)).

Most neurodegenerative diseases affect neuronal populations.
Moreover, most of the damage occurs to a specific
neurochemical phenotype. In human Parkinson's disease, for
example, the major cell type lost is midbrain dopaminergic
neurons. Functional replacement of specific neuronal
populations through transplantation of neural tissue
represents an attractive therapeutic strategy for treating

neurodegenerative diseases (Rosenthal, A. Neuron 20, 169-172
(1998)).

Summary of the Invention

5   An ideal material for use in transplantation therapy is an
expandable cell that could be instructed to assume a neuronal
phenotype, particularly a specific neuronal phenotype, upon
differentiation. This strategy would circumvent ethical and
practical issues surrounding the use of human fetal tissue for
10   transplantation. In particular, implanted embryonic cells are
of limited viability and are often rejected. In addition,
each fetus provides only a small number of cells.

Induction of a single and specific neuronal phenotype in
15   multipotent neural stem or progenitor cells *in vitro* has
proven elusive.

In a first aspect, the present invention provides a method of
inducing a neuronal fate in a neural stem cell or neural
20   progenitor cell, the method including expressing *Nurr1* above
basal levels within the cell.

Nurr1 (Law, et al., (1992) Mol Endocrinol 6, 2129-2135; Xing,
et al., (1997) Mol Brain Res 47, 251-261; Castillo (1997)
25   Genomics, 41, 250-257; GenBank nos. S53744, U72345, U86783) is
a transcription factor of the thyroid hormone/retinoic acid
nuclear receptor superfamily. The present disclosure shows
that expression of *Nurr1* above basal levels in neural stem
cells or neural progenitor cells increases the proportion of
30   the cells which differentiate toward a neuronal fate. The
induction of a neuronal fate may be carried out in vitro or in
vivo. The ability to induce differentiation of neural stem or
progenitor cells toward the neuronal fate prior to, or
following transplantation ameliorates the biasing of
35   transplanted stem cells to the astro- and oligodendroglial
fates.

By "neural stem cell" is meant any cell type that can divide more than once and can give rise to cells that exhibit the most primitive type of phenotypes for neurons, astrocytes and
5   oligodendrocytes. The neural stem cell may express one or more of the following markers: Nestin; the p75 neurotrophin receptor; Notch1. By "neural progenitor cell" is meant a multipotent daughter of a neural stem cell, which daughter is restricted in its potential fates and/or has a reduced
10   proliferative potential compared to the neural stem cell. In preferred embodiments, the neural stem or progenitor cell does not express tyrosine hydroxylase either spontaneously or upon deprivation of mitogens (e.g. bFGF, EGF or serum).

15   The neural stem cell or neural progenitor cell may be obtained or derived from any region of the nervous system, e.g. from the cerebellum, the ventricular zone, the sub-ventricular zone or the hippocampus. It may be obtained or derived from a vertebrate organism, e.g. from a mammal, which may be human
20   or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle, horse, or primate, or from a bird, such as a chicken.

The neuronal fate to which the neural stem or progenitor cell
25   is induced may exhibit a primitive neuronal phenotype. It may be a multipotent cell which is capable of giving rise to a plurality of distinct neuronal phenotypes. It may lack markers associated with specific neuronal fates, e.g. tyrosine hydroxylase.

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In a method of inducing a neuronal fate according to the present invention wherein a plurality of neural stem cells and/or progenitor cells express Nurr1 above basal levels, a majority of the stem or progenitor cells may be induced to
35   adopt a neuronal fate. In preferred embodiments, more than 60%, more than 70%, more than 80%, more than 90% of the stem and/or progenitor cells may be induced to a neuronal fate.

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By "expressing *Nurr1* above basal levels within the cell" is meant expressing *Nurr1* at levels greater than that at which it is expressed in the (unmodified) cell *in vivo* under non-pathological conditions. Expression above basal levels

5   includes pharmacological and artificial upregulation and over-expression.

Expression of *Nurr1* above basal levels may be achieved by any method known to those skilled in the art. By way of example,

10   expression above basal levels may be induced by modulating the regulation of native genomic *Nurr1*. This may be done by increasing transcription and/or translation of *Nurr1*, e.g. by contacting the cell with fibroblast growth factor 8 (FGF8), which upregulates transcription of *Nurr1* (Rosenthal, A.,

15   (1998) Cell, *93(5)*,755-766), and/or by introducing heterologous regulatory sequences into or adjacent the native regulatory region of *Nurr1*, and/or by replacing the native regulatory region of *Nurr1* with such heterologous regulatory sequences, e.g. by homologous recombination, and/or by

20   disrupting or downregulating molecules that negatively regulate, block or downregulate transcription, translation or the function of *Nurr1*, e.g. *Nurr2* (Ohkura, et al., (1999) Biochim Biophys Acta 14444: 69-79).

25   Transcription may be increased by providing the neural stem or progenitor cell with increased levels of a transcriptional activator, e.g. by contacting the cell with such an activator or by transformation of the cell with nucleic acid encoding the activator. Alternatively, transcription may be increased

30   by transforming the cell with antisense nucleic acid to a transcriptional inhibitor of *Nurr1*.

Accordingly, a method of the present invention of inducing a neuronal fate in a neural stem or progenitor cell, may include

35   contacting the cell with FGF8.

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As an alternative or addition to increasing transcription and/or translation of endogenous *Nurr1*, expression of *Nurr1* above basal levels may be caused by introduction of one or more extra copies of *Nurr1* into the neural stem or progenitor

5   cell.

Accordingly, in a further aspect, the present invention provides a method of inducing a neuronal fate in a neural stem cell or neural progenitor cell, the method including

10   transforming the cell with *Nurr1*. Transformation of the neural stem or progenitor cell may be carried out in vitro or in vivo. The neuronal fate to which the neural stem or progenitor cell is induced may be of the type discussed herein, e.g. it may exhibit a primitive neuronal phenotype and

15   may lack markers associated with specific neuronal fates. The invention further provides a neural stem cell or neural progenitor cell transformed with *Nurr1*.

Transformed *Nurr1* may be contained on an extra-genomic vector

20   or it may be incorporated, preferably stably, into the genome. It may be operably-linked to a promotor which drives its expression above basal levels in neural stem or progenitor cells, as is discussed in more detail below.

25   "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter.

Methods of introducing genes into cells are well known to

30   those skilled in the art. Vectors may be used to introduce *Nurr1* into neural stem or progenitor cells, whether or not the *Nurr1* remains on the vector or is incorporated into the genome. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including

35   promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences. Vectors may contain marker

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genes and other sequences as appropriate. The regulatory sequences may drive expression of *Nurr1* within the neural stem or progenitor cells. For example, the vector may be an extra-genomic expression vector, or the regulatory sequences may be

5   incorporated into the genome with *Nurr1*. Vectors may be plasmids or viral.

Nurr1 may be placed under the control of an externally inducible gene promoter to place it under the control of the

10   user. The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some

15   inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

20   An example of an inducible promoter is the Tetracyclin ON/OFF system (Gossen, et al., (1995) Science, *268*, 1766-1769) in which gene expression is regulated by tetracyclin analogs.

For further details see, for example, Molecular Cloning: a

25   Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene

30   expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Marker genes such as antibiotic resistance or sensitivity

35   genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art. Clones may also be identified or further investigated by binding studies, e.g.

by Southern blot hybridisation.

Nucleic acid including *Nurr1* may be integrated into the genome
of the host neural stem cell or neural progenitor cell.

5   Integration may be promoted by including in the transformed
nucleic acid sequences which promote recombination with the
genome, in accordance with standard techniques. The
integrated nucleic acid may include regulatory sequences able
to drive expression of the *Nurr1* gene in neural stem or

10   progenitor cells. The nucleic acid may include sequences
which direct its integration to a site in the genome where the
Nurr1 coding sequence will fall under the control of
regulatory elements able to drive and/or control its
expression within the neural stem or progenitor cell. The

15   integrated nucleic acid may be derived from a vector used to
transform *Nurr1* into the neural stem or progenitor cells, as
discussed herein.

The introduction of nucleic acid comprising *Nurr1*, whether

20   that nucleic acid is linear, branched or circular, may be
generally referred to without limitation as "transformation".
It may employ any available technique. Suitable techniques
may include calcium phosphate transfection, DEAE-Dextran,
electroporation, mechanical techniques such as microinjection,

25   direct DNA uptake, receptor mediated DNA transfer,
transduction using retrovirus or other virus and liposome-
mediated transfection. When introducing a chosen gene
construct into a cell, certain considerations must be taken
into account, well known to those skilled in the art. It will

30   be apparent to the skilled person that the particular choice
of method of transformation to introduce *Nurr1* into neural
stem or progenitor cells is not essential to or a limitation
of the invention.

35   Suitable vectors and techniques for in vivo transformation of
neural stem or progenitor cells with *Nurr1* are well known to

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those skilled in the art. Suitable vectors include adenovirus, papovavirus, vaccinia virus, herpes virus and retroviruses. Disabled virus vectors may be produced in helper cell lines in which genes required for production of

5   infectious viral particles are expressed. Suitable helper cell lines are well known to those skilled in the art. By way of example, see: Fallaux, F.J., et al., (1996) Hum Gene Ther 7(2), 215-222; Willenbrink, W., et al., (1994) J Virol 68(12), 8413-8417; Cosset, F.L., et al., (1993) Virology 193(1), 385-

10   395; Highkin, M.K., et al., (1991) Poult Sci 70(4), 970-981; Dougherty, J.P., et al., (1989) J Virol 63(7), 3209-3212; Salmons, B., et al., (1989) Biochem Biophys Res Commun 159(3), 1191-1198; Sorge, J., et al., (1984) Mol Cell Biol 4(9), 1730-1737; Wang, S., et al., (1997) Gene Ther 4(11), 1132-1141;

15   Moore, K.W., et al., (1990) Science 248(4960), 1230-1234; Reiss, C.S., et al., (1987) J Immunol 139(3), 711-714. Helper cell lines are generally missing a sequence which is recognised by the mechanism which packages the viral genome. They produce virions which contain no nucleic acid. A viral

20   vector which contains an intact packaging signal along with the gene or other sequence to be delivered (*Nurr1*) is packaged in the helper cells into infectious virion particles, which may then be used for gene delivery to neural stem or progenitor cells.

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In a further aspect, the present invention provides a method of inducing a specific neuronal fate in a neural stem or progenitor cell, wherein the stem cell or progenitor cell expresses *Nurr1* above basal levels, the method including

30   contacting the cells with one or more factors supplied by or derived from a Type 1 astrocyte. The factor or factors may be provided by co-culturing the neural stem cell or neural progenitor cell with a Type 1 astrocyte. The method may occur in vitro or in vivo. The neural stem or progenitor cells

35   expressing *Nurr1* above basal levels may be produced by transformation of the cells with *Nurr1*.

The factor or factors may be supplied by or derived from an immortalized astrocyte. The factor or factors may be supplied by or derived from an astrocyte cell line, e.g. an astrocyte mesencephalic cell line. Cell lines provide a homogenous cell

5   population.

The present disclosure provides the first evidence that Type 1 astrocytes are involved in the determination of specific neuronal fates. The data presented herein suggests that

10   astrocytes from distinct brain regions may be used as a source of signals required for the induction of regionally appropriate neuronal phenotypes in multiple brain structures.

Important aspects of the present invention are based on the

15   finding that dopaminergic neurons can be generated from multipotent neural stem cells or progenitor cells *in vitro* by a process including expression of *Nurr1* above basal levels in the cells and contact of the cells with one or more factors supplied by or derived from Type 1 astrocytes of the ventral

20   mesencephalon.

Accordingly, the specific neuronal fate is preferably a dopaminergic fate, and the Type 1 astrocyte is preferably a Type 1 astrocyte of the ventral mesencephalon.

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The present invention allows for generation of large numbers of dopaminergic neurons. These dopaminergic neurons may be used as source material to replace cells which are damaged or lost in Parkinson's disease.

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Preferably, the neural stem or progenitor cell expressing *Nurr1* above basal levels is mitotic when it is contacted with the one or more factors supplied by or derived from the Type 1 astrocyte.

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In inducing a dopaminergic fate in a neural stem or progenitor cell, the cell may additionally be contacted with one or more

agents selected from: basic fibroblast growth factor (bFGF);
epidermal growth factor (EGF); and an activator of the
retinoid X receptor (RXR), e.g. the synthetic retinoid analog
SR11237, (Gendimenico, G. J., et al., (1994) J Invest Dermatol

5   *102(5)*, 676-80), or 9-cis retinol. Treating a co-culture of
neural stem and/or progenitor cells expressing *Nurr1* above
basal levels and Type 1 astrocytes with one or more of these
agents may be used to increase the proportion of the stem
and/or progenitor cells which adopt a dopaminergic fate, as

10   demonstrated experimentally below. The method of inducing a
dopaminergic fate in accordance with the present invention may
include contacting the neural stem or progenitor cell with a
member of the FGF family of growth factors, e.g. FGF4, FGF8.

15   Advantageously, the cells may be contacted with two or more of
the above agents. The inventors have unexpectedly found that
the beneficial effects of bFGF or EGF and SR11237 are additive
at saturating doses. This finding suggests that these agents
may act through different mechanisms.

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The method of inducing a dopaminergic phenotype may include
pretreating the neural stem cell or neural progenitor cell
with bFGF and/or EGF prior to contacting it with the one or
more factors supplied by or derived from Type 1 astrocytes of

25   the ventral mesencephalon, e.g. prior to co-culturing it with
a Type 1 astrocyte of the ventral mesencephalon.

The optional pretreatment step arises from two further
unexpected findings of the inventors: (i) that neural stem

30   cell lines expressing *Nurr1* above basal levels and showing
high proliferation demonstrate enhanced induction to
dopaminergic fate when co-cultured with Type 1 astrocytes of
the ventral mesencephalon; and (ii) that after treatment with
bFGF or EGF in serum-free medium (SFM), the baseline

35   proliferation of most stem cell lines expressing *Nurr1* above
basal levels remained elevated after passage into SFM alone.

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The method of inducing a dopaminergic phenotype may include
pretreating a neural stem cell or progenitor cell with a
member of the FGF family of growth factors, e.g. FGF4, FGF8.

5   A method according to the invention in which a neuronal fate
     is induced in a multipotent neural stem or progenitor cell may
     include detecting a marker for the neuronal fate. β-tubulin
     III (TuJ1) is one marker of the neuronal fate (Menezes, J. R.,
     et al., (1994) J Neurosci 14(9), 5399-5416). If a particular
10   neuronal phenotype is induced, the marker should be specific
     for that phenotype. For the dopaminergic fate, expression of
     tyrosine hydroxylase (TH) and/or release of dopamine and/or
     DOPAC may be detected e.g. by immunoreactivity (Cooper, J. R.,
     et al., The Biochemical Basis of Neuropharmacology, 7th
15   Edition, (1996) Oxford University Press). The absence of
     Dopamine β hydroxylase (in the presence of TH/dopamine/DOPAC)
     is also indicative of dopaminergic fate.

     Detection of a marker may be carried out according to any
20   method known to those skilled in the art. The detection method
     may employ a specific binding member capable of binding to a
     nucleic acid sequence encoding the marker, the specific
     binding member comprising a nucleic acid probe hybridisable
     with the sequence, or an immunoglobulin/antibody domain with
25   specificity for the nucleic acid sequence or the polypeptide
     encoded by it, the specific binding member being labelled so
     that binding of the specific binding member to the sequence or
     polypeptide is detectable. A "specific binding member" has a
     particular specificity for the marker and in normal conditions
30   binds to the marker in preference to other species.
     Alternatively, where the marker is a specific mRNA, it may be
     detected by binding to specific oligonucleotide primers and
     amplification in e.g. the polymerase chain reaction.

35   Nucleic acid probes and primers may hybridize with the marker
     under stringent conditions. Suitable conditions include, e.g.
     for detection of marker sequences that are about 80-90%

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identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4, pH
7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in
0.1X SSC, 0.1% SDS. For detection of marker sequences that
are greater than about 90% identical, suitable conditions

5   include hybridization overnight at 65°C in 0.25M Na_2HPO_4, pH
7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in
0.1X SSC, 0.1% SDS.

In a further aspect, the present invention provides a neuron
10   produced in accordance with any one of the methods disclosed
herein. The neuron may have a primitive neuronal phenotype.
It may be capable of giving rise to a plurality of distinct
neuronal phenotypes. The neuron may have a particular
neuronal phenotype, the phenotype being influenced by the type
15   of astrocyte from which the factor or factors which contacted
the neural stem or progenitor cell expressing Nurr1 above
basal levels were supplied or derived, or by the type of
astrocyte with which the stem or progenitor cell was co-
cultured. In preferred embodiments, the neuron has a
20   dopaminergic phenotype.

The neuron may contain nucleic acid encoding a molecule with
neuroprotective or neuroregenerative properties operably
linked to a promoter which is capable of driving expression of
25   the molecule in the neuron. The promoter may be an inducible
promoter, e.g. the TetON chimeric promoter, so that any
damaging over-expression may be prevented. The promoter may
be associated with a specific neuronal phenotype, e.g. the TH
promoter.
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The encoded molecule may be such that its expression renders
the neuron independent of its environment, i.e. such that its
survival is not dependent on the presence of one or more
factors or conditions in e.g. the neural environment into
35   which it is to be implanted. By way of example, the neuron
may contain nucleic acid encoding one or more of the
neuroprotective or neuroregenerative molecules described below

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operably linked to a promoter which is capable of driving
expression of the molecule in the neuron.

In addition or alternatively, expression of the encoded
5   molecule may function in neuroprotection or neuroregeneration
of the cellular environment surrounding that neuron. In this
way, the neuron may be used in a combined cell and gene
therapy approach to deliver molecules with neuroprotective and
neuroregenerative properties.

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Examples of molecules with neuroprotective and
neuroregenerative properties include:

(i) neurotropic factors able to compensate for and prevent
15   neurodegeneration. One example is glial derived neurotropic
growth factor (GDNF) which is a potent neural survival factor,
promotes sprouting from dopaminergic neurons and increases
tyrosine hydroxylase expression (Tomac, et al.,(1995) Nature,
373, 335-339; Arenas, et al., (1995) Neuron, 15,1465-1473).

20   By enhancing axonal elongation GDNF, GDNF may increase the
ability of the neurons to inervate their local environment.
Other neurotropic molecules of the GDNF family include
Neurturin, Persephin and Artemin. Neurotropic molecules of
the neurotropin family include nerve growth factor (NGF),
25   brain derived neurotropic factor (BDNF), and neurotropin-3, -
4/5 and -6.

(ii) antiapoptotic molecules. Bcl2 which plays a central
role in cell death. Over-expression of Bcl2 protects neurons
30   from naturally occurring cell death and ischemia (Martinou, et
al., (1994) Neuron, 1017-1030). Another antiapoptotic
molecule is BclX-L.

(iii) axon regenerating and/or elongating and/or guiding
35   molecules which assist the neuron in innervating and forming
connections with its environment, e.g. ephrins. Ephrins
define a class of membrane-bound ligands capable of activating

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tyrosine kinase receptors. Ephrins have been implicated in neural development (Irving, et al., (1996) Dev. Biol., *173*, 26-38; Krull, et al., (1997) Curr. Biol. 7, 571-580; Frisen, et al., (1998) Neuron, *20*, 235-243; Gao, et al., (1996) PNAS,

5   *93*, 11161-11166; Torres, et al., (1998) Neuron, *21*, 1453-1463; Winslow, et al., (1995) Neuron, *14*, 973-981; Yue, et al., (1999) J Neurosci *19(6)*, 2090-2101.

(iv) differentiation molecules, e.g. the homeobox domain
10   protein Ptx3 (Smidt, M. P., et al., (1997) Proc Natl Acad Sci USA, *94(24)*, 13305-13310).

A neuron in accordance with or for use in the present invention may be substantially free from one or more other
15   cell types, e.g. from neural stem or progenitor cells. Neurons may be separated from neural stem or progenitor cells using any technique known to those skilled in the art. By way of example, antibodies against extracellular regions of molecules found on neural stem or progenitor cells but not on
20   neurons may be employed. Such molecules include Notch 1 and the glial cell line derived neurotrophic factor receptor GFR α2. Stem cells bound to antibodies may be lysed by exposure to complement, or separated by, e.g. magnetic sorting (Johansson, et al., (1999) Cell, *96*, 25-34). If antibodies
25   which are xenogeneic to the intended recipient of the neurons are used, then any e.g. neural stem or progentior cells which escape such a cell sorting procedure are labelled with xenogeneic antibodies and are prime targets for the recipient's immune system.
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The present invention extends in various aspects not only to a neuron produced in accordance with any one of the methods disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a
35   neuron, use of such a neuron or composition in a method of medical treatment, a method comprising administration of such

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a neuron or composition to a patient, e.g. for treatment (which may include preventative treatment) of Parkinson's disease or other (e.g. neurodegenerative) diseases, use of such a neuron in the manufacture of a composition for

5 administration, e.g. for treatment of Parkinson's disease or other (e.g. neurodegenerative diseases), and a method of making a pharmaceutical composition comprising admixing such a neuron with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally one or more other ingredients, e.g.

10 a neuroprotective molecule, a neuroregenerative molecule, a retinoid, growth factor, astrocyte, anti-apoptotic factor, or factor that regulates gene expression in neural stem or progenitor cells or in the host brain. Such optional ingredients may render the neuron independent of its

15 environment, i.e. such that its survival is not dependent on the presence of one or more factors or conditions in its environment. By way of example, the method of making a pharmaceutical composition may include admixing the neuron with one or more factors found in the developing ventral

20 mesencephalon. The neuron may be admixed with GDNF and/or neurturin (NTN).

The present invention provides a composition containing a neuron produced in accordance with the invention, and one or

25 more additional components. Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to the neuron, a pharmaceutically acceptable excipient, carrier, buffer, preservative, stabiliser, anti-oxidant or other

30 material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the activity of the neuron. The precise nature of the carrier or other material will depend on the route of administration. The composition may include one or more of a neuroprotective

35 molecule, a neuroregenerative molecule, a retinoid, growth factor, astrocyte, or factor that regulates gene expression in neural stem or progenitor cells. Such substances may render the neuron independent of its environment as discussed above.

Liquid pharmaceutical compositions generally include a liquid
carrier such as water, petroleum, animal or vegetable oils,
mineral oil or synthetic oil. Physiological saline solution,
dextrose or other saccharide solution or glycols such as

5   ethylene glycol, propylene glycol or polyethylene glycol may
be included.

The composition may be in the form of a parenterally
acceptable aqueous solution which is pyrogen-free and has

10   suitable pH, isotonicity and stability. Those of relevant
skill in the art are well able to prepare suitable solutions
using, for example, isotonic vehicles such as Sodium
Chloride, Ringer's Injection, or Lactated Ringer's Injection.
A composition may be prepared using artificial cerebrospinal

15   fluid.

The present invention extends to the use of a neuron produced
in accordance with the invention in a method of medical
treatment, particularly the treatment of a medical condition

20   associated with damage to, the loss of, or a disorder in,
neuronal cells. Moreover, the invention may provide the use
of a neuron of a specific phenotype in the treatment of a
condition, disease or disorder which is associated with damage
to, or the loss of neurons of that phenotype. More

25   particularly, the invention provides the use of a dopaminergic
neuron in the treatment of human Parkinson's disease. While
the invention particularly relates to materials and methods
for treatment of neurodegenerative diseases (e.g. Parkinson's
disease), it is not limited thereto. By way of example, the

30   invention extends to the treatment of damage to the spinal
cord and/or cerebral cortex.

In methods of treatment in which the administered neuron is a
pluripotent cell that is capable of giving rise to two or more

35   distinct neuronal phenotypes, the neuron or composition may be
introduced into a region containing astrocytes which direct
the differentiation of the neuron to a desired specific
neuronal fate. The neuron or composition may for example be

injected into the ventral mesencephalon where it may interact
with Type 1 astrocytes and be induced to adopt a dopaminergic
phenotype. Alternatively or in addition, an implanted
composition may contain a pluripotent neuron in combination
5   with one or more factors which direct its development toward a
specific neuronal fate as discussed above, e.g. with a Type 1
astrocyte.

Neurons may be implanted into a patient by any technique known
10   in the art (e.g. Lindvall, O., (1998) Mov. Disord. *13*, Suppl.
1:83-7; Freed, C.R., et al., (1997) Cell Transplant, *6*, 201-
202; Kordower, et al., (1995) New England Journal of Medicine,
332, 1118-1124; Freed, C.R.,(1992) New England Journal of
Medicine, 327, 1549-1555).

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Administration of a composition in accordance with the present
invention is preferably in a "prophylactically effective
amount" or a "therapeutically effective amount" (as the case
may be, although prophylaxis may be considered therapy), this
20   being sufficient to show benefit to the individual. The
actual amount administered, and rate and time-course of
administration, will depend on the nature and severity of what
is being treated. Prescription of treatment, e.g. decisions
on dosage etc, is within the responsibility of general
25   practitioners and other medical doctors.

A composition may be administered alone or in combination with
other treatments, either simultaneously or sequentially
dependent upon the condition to be treated.

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The methods provided herein for inducing a neuronal fate in
neural stem or progenitor cells may be carried out using
neural stem cell lines or neural progenitor cell lines as a
source material. In this way there is virtually no limitation
35   on the number of neurons which may be produced.

In order to ameliorate possible disadvantages associated with

immunological rejection of transplanted cells, neural stem or progenitor cells may be isolated from a patient and induced to the desired phenotype. Advantageously, isolated neural stem or progenitor cells may be used to establish stem cell or

5 progenitor cell lines so that large numbers of immunocompatible neuronal cells may be produced. A further option is to establish a bank of cells covering a range of immunological compatibilities from which an appropriate choice can be made for an individual patient. Neural stem or

10 progenitor cells derived from one individual may be altered to ameliorate rejection when they or their progeny are introduced into a second individual. By way of example, one or more MHC alleles in a donor cell may be replaced with those of a recipient, e.g. by homologous recombination.

15

If neural stem or progenitor cells derived from a cell line carrying an immortalizing oncogene are used for implantation into a patient, the oncogene may be removed using the CRE-loxP system prior to implantation of the cells into a patient

20 (Westerman, K. A. et al Proc. Natl. Acad Sci. USA 93, 8971 (1996)). An immortalizing oncogene which is inactive at the body temperature of the patient may be used.

In a further aspect the present invention extends to the use

25 of a neuron produced in accordance with the invention in a method of screening for an agent for use in the treatment of a neurodegenerative disease. The neuron may be a dopaminergic neuron. The neurodegenerative disease may be Parkinson's disease. The agent may be a neuroprotective and/or

30 neuroregenerative molecule. The method may be carried out *in vitro*.

The method may include:
(i) treating a neuron of the invention with a toxin for said

35 neuron;
(ii) separating the neuron from the toxin;
(iii) bringing the treated neuron into contact with a test

19

agent or test agents;

(iv) determining the ability of the neuron to recover from the toxin;

(v) comparing said ability of the neuron to recover from the toxin with the ability of the or an identical neuron to recover from the toxin in the absence of contact with the test agent(s).

The method may include:

(i) treating a neuron of the invention with a toxin for the neuron in the presence of a test agent or test agents;

(ii) determining the ability of the neuron to tolerate the toxin;

(iii) comparing said ability of the neuron to tolerate the toxin with the ability of the or an identical neuron to tolerate the toxin in the absence of contact with the test agent(s).

The toxin may be 6-hydroxydopamine, 5,7-dihydroxytryptamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The ability of the neuron to recover from or tolerate the toxin may be determined by any method known to those skilled in the art, for example by monitoring cell viability, (e.g. by cell counting, e.g. by the TUNEL technique), by monitoring morphology, (e.g. sprouting, axonal elongation and/or branching), and/or by monitoring biochemistry, (e.g. TH activity, e.g. neurotransmitter uptake/release/content).

Neural stem or progenitor cells which may be used in the present invention include C17.2 (Snyder, E. Y. et al. Cell 68, 33-51 (1992)) and the H6 human cell line (Flax et al. Nature Biotech 16 (1998)). Further examples are listed in (Gage, F.H. et al. Ann. Rev. Neurosci. 18, 159-192 (1995)).

While the present discussion has been made with reference to neural stem cells or neural progenitor cells, the methods provided herein may be applied to the induction of neuronal fates in other stem/progenitor cells. Examples of such cells

include stem cells associated with non-neural systems. The methods may be applied to hematopoietic stem cells and/or proliferative cells from the epidermis. Hematopoietic cells may be collected from blood or bone biopsy. Epithelial cells

5   may be collected by skin biopsy or by scraping e.g. the oral mucosa. Since a neuronal phenotype is not a physiological *in vivo* fate of these stem/progenitor cells, the inductive process may be referred to as trans-differentiation, or desdifferentiation and neural redifferentiation. A method of

10   inducing such cells to a neuronal fate may include the use of antisense regulators to genes associated with non-neuronal phenotypes, i.e. to suppress and/or reverse the differentiation of these cells toward non-neuronal fates.

15   The methods of the present invention may be applied to stem cells located before neural stem cells in the developmental pathway. They may be applied to stem cells which are capable of giving rise to two or more daughter stem cells associated with different developmental systems. Examples of daughter

20   stem cells are hematopoietic stem cells, proliferative cells from the epidermis, and neural stem cells.

As discussed above, the present disclosure demonstrates that dopaminergic neurons can be generated from multipotent neural

25   stem or progenitor cells by a process requiring expression of *Nurr1* above basal levels in combination with one or more factors supplied by or derived from Type 1 astrocytes of the ventral mesencephalon. The one or more factors are soluble and secreted, as determined by a capacity to pass through a

30   microporous insert. The factor or factors also appear to be labile.

In various further aspects the present invention is concerned with provision of assays and methods of screening for a factor

35   or factors which induce a dopaminergic fate in a neural stem or progenitor cell expressing *Nurr1* above basal levels, and with a factor or factors identified thereby.

The invention provides a method of screening for a factor or factors able, either alone or in combination, to induce a dopaminergic fate in a neural stem or progenitor cell expressing *Nurr1* above basal levels. A further aspect of the

5 present invention provides the use of a neural stem or progenitor cell expressing *Nurr1* above basal levels in screening or searching for and/or obtaining/identifying a factor or factors which induce(s) a dopaminergic fate in such a stem or progenitor cell.

10

A method of screening may include:

(a) bringing a test substance into contact with a neural stem or progenitor cell expressing *Nurr1* above basal levels, which contact may result in interaction between the test

15 substance and the neural stem or progenitor cell; and

(b) determining interaction between the test substance and the stem or progenitor cell.

A method of screening may include bringing a test substance

20 into contact with a membrane fraction derived from a neural stem or progenitor cell expressing *Nurr1* above basal levels and determining interaction between the test substance and the membrane fraction. The preparation of membrane fractions is well within the capabilities of those skilled in the art.

25

Binding or interaction may be determined by any number of techniques known in the art, qualitative or quantitative. Interaction between the test substance and the stem or progenitor cell may be studied by labeling either one with a

30 detectable label and bringing it into contact with the other which may have been immobilised on a solid support, e.g. by using an antibody bound to a solid support, or via other technologies which are known *per se*.

35 A screening method may include culturing a neural stem or progenitor cell in the presence of a test substance or test substances and analyzing the cell for differentiation to a

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dopaminergic phenotype, e.g. by detecting a marker of the
dopaminergic phenotype as discussed herein. Tyrosine
hydroxylase (TH) is one marker of the dopaminergic phenotype.

5   Any of the substances screened in accordance with by the
present invention may be a natural or synthetic chemical
compound.

A screening method may include comparing Type 1 astrocytes of
10   the ventral mesencephalon with neural cells (e.g. astrocytes)
which are unable to induce a dopaminergic fate in neural stem
or progenitor cells expressing Nurr1 above basal levels. The
comparison may for example be between Type 1 astrocytes of the
ventral mesencephalon and Type 1 astrocytes from other neural
15   locations.

A screening method involving astrocytes may employ
immortalized astrocytes. It may involve astrocyte cell lines,
e.g. astrocyte mesencephalic cell lines. Such cell lines
20   provide a homogenous cell population.

A screening method may employ any known method for analyzing a
phenotypic difference between cells and may be at the DNA,
mRNA, cDNA or polypeptide level. Differential screening and
25   gene screening are two such techniques. A substance
identified by any of the methods of screening described herein
may be used as a test substance in any of the other screening
methods described herein.

30   A screening method may employ a nucleic expression array, e.g.
a mouse cDNA expression array. In this approach, an array of
different nucleic acid molecules is arranged on a filter, e.g.
by cross-linking the nucleic acid to the filter. A test
solution or extract is obtained and the nucleic acid within it
35   is labeled, e.g. by fluorescence. The solution or extract is
then applied to the filter. Hybridisation of the test nucleic
acid to nucleic acid on the filter is determined and compared

to the hybridisation achieved with a control solution. A
difference between the hybridisation obtained with the test
and control samples is indicative of a different nucleic acid
content. For further information on nucleic acid arrays, see
5   www.clontech.com.

A screening method may include comparing C17.2 parental cells
with C17.2 cells which express *Nurr1* above basal levels, e.g.
to identify target genes of Nurr1 and/or the receptor(s) for
10   the factor or factors which are supplied by or derived from
Type 1 astrocytes of the ventral mesencephalon and which
induce a dopaminergic fate in neural stem or progenitor cells
expressing *Nurr1* above basal levels. Once the target gene(s)
and/or receptor(s) have been identified they may be isolated
15   and/or purified and/or cloned and used in methods of screening
for the factor or factors themselves, e.g. by affinity
chromatography.

A screening method may include purifying and/or isolating a
20   substance or substances from a mixture. The method may
include determining the ability of one or more fractions of
the mixture to interact with a neural stem cell or neural
progenitor cell expressing *Nurr1* above basal levels, e.g. the
ability to bind to and/or induce a dopaminergic fate in such a
25   neural stem or progenitor cell. The purifying and/or isolating
may employ any method known to those skilled in the art.

A screening method may employ an inducible promoter operably
linked to nucleic acid encoding a test substance. Such a
30   construct is incorporated into a host cell and one or more
properties of that cell under the permissive and non-
permissive conditions of the promoter are determined and
compared. The property determined may be the ability of the
host cell to induce a dopaminergic phenotype in a co-cultured
35   neural stem or progenitor cell expressing *Nurr1* above basal
levels. A difference in that ability of the host cell between
the permissive and non-permissive conditions indicates that

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the test substance may be able, either alone or in combination, to induce a dopaminergic fate in a neural stem or progenitor cell expressing *Nurr1* above basal levels.

5   The precise format of any of the screening methods of the present invention may be varied by those of skill in the art using routine skill and knowledge.

A factor or factors identified by any one of the methods
10 provided by the invention may be isolated and/or purified and/or further investigated. It may be manufactured.

In various further aspects, the invention further provides a factor identified by any one of the methods disclosed herein,
15 a pharmaceutical composition, medicament, drug or other composition comprising such a factor (which composition may include a neural stem or progenitor cell expressing *Nurr1* above basal levels), use of such a factor to induce neural stem or progenitor cells expressing *Nurr1* above basal levels
20 to adopt a dopaminergic phenotype, use of such a factor or composition in a method of medical treatment, a method comprising administration of such a factor or composition to a patient, e.g. for treatment (which may include preventative treatment) of a medical condition associated with damage to,
25 loss of, or a disorder in dopaminergic neurons, e.g. for treatment of Parkinson's disease or another neurodegenerative disease, use of such a factor in the manufacture of a composition, medicament or drug for administration, e.g. for treatment of Parkinson's disease or other (e.g.
30 neurodegenerative diseases), and a method of making a pharmaceutical composition comprising admixing such a factor with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

35 In a related aspect, the present invention provides a method of screening for a substance which modulates the ability of Type 1 astrocytes of the ventral mesencephalon (or of a factor

or factors identified by a screening method provided by the invention) to induce a dopaminergic fate in neural stem or progenitor cells expressing *Nurr1* above basal levels.

5 Such a method may include one or more of:
(i) co-culturing Type 1 astrocytes with neural stem or progenitor cells which express *Nurr1* above basal levels in the presence of one or more test substances;
(ii) bringing neural stem or progenitor cells which express
10 *Nurr1* above basal levels into contact with one or more factors identified by a screening method provided by the invention as being able to induce a dopaminergic phenotype in such cells, said contact occurring in the presence of one or more test substances;
15 (iii) analysing the proportion of stem or progenitor cells which adopt a dopaminergic fate;
(iv) comparing the proportion of stem or progenitor cells which adopt a dopaminergic fate with the number of stem or progenitor cells which adopt a dopaminergic fate in comparable
20 reaction medium and conditions in the absence of the test substance(s). A difference in the proportion of dopaminergic neurons between the treated and untreated co-cultures is indicative of a modulating effect of the relevant test substance(s).
25

Such a method of screening may include:
(i) co-culturing Type 1 astrocytes with neural stem or progenitor cells which express *Nurr1* above basal levels in the presence of one or more test substances;
30 (ii) analysing the proportion of stem or progenitor cells which adopt a dopaminergic fate;
(iii) comparing the proportion of stem or progenitor cells which adopt a dopaminergic fate with the number of stem or progenitor cells which adopt a dopaminergic fate in comparable
35 reaction medium and conditions in the absence of the test substance(s).

Such a method of screening may include:

(i) bringing neural stem or progenitor cells which express *Nurr1* above basal levels into contact with one or more factors identified by a screening method provided by the invention as
5   being able to induce a dopaminergic phenotype in such cells, said contact occurring in the presence of one or more test substances;

(ii) analysing the proportion of stem or progenitor cells which adopt a dopaminergic fate;
10   (iii) comparing the proportion of stem or progenitor cells which adopt a dopaminergic fate with the number of stem or progenitor cells which adopt a dopaminergic fate in comparable reaction medium and conditions in the absence of the test substance(s).
15

Following identification of a substance which modulates inductive activity, the substance may be investigated further. It may be manufactured and/or used in the preparation, i.e. manufacture or formulation, of a composition such as a
20   medicament, pharmaceutical composition or drug. Any of substance tested for its modulating activity may be a natural or synthetic chemical compound.

Aspects and embodiments of the present invention will now be
25   illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this specification are incorporated herein by reference.

30   **Brief Description of the Figures**

Figure 1 demonstrates the characterization of the *Nurr1*-C17.2 clones.
Figure 1A shows the residual proliferative rate of the
35   parental-, *Nurr1*- and mock-C17.2 control clones. %BrdU+ indicates the percentage of cells which incorporated BrdU after a six-hour pulse in serum free media.

<u>Figure 1B</u> shows that expression of *Nurr1* significantly

increases neuronal fate in serum free media. %TuJ1+/%BrdU-

indicates the percentage of cells which expressed β-tubulin

III but did not incorporate BrdU.

5   <u>Figure 1C</u> shows that co-culturing two of the clones with E16

ventral mesencephalic cells significantly increases

dopaminergic fate. %TH+ indicates the percentage of tyrosine

hydroxylase positive C17.2 cells.

10   <u>Figure 2</u> demonstrates the role of retinoids, bFGF, and

proliferation in the induction of dopaminergic neurons from

Nurr1-over-expressing neural stem or progenitor cell lines in

ventral mesencephalic co-cultures.

<u>Figure 2A</u> shows the effects of SR11237, bFGF, and EGF on

15   dopaminergic induction. %TH+ indicates the percentage of C17.2

cells in co-culture expressing tyrosine hydroxylase. (C) co-

cultures of E16 VM cells with parental C17.2 cells or with

Nurr1-C17.2-clone 42 cells; (SR) co-culture plus SR11237; (F)

co-culture plus bFGF; (αF) co-culture plus blocking antibody

20   to bFGF (E) co-culture plus EGF.

<u>Figure 2B</u> demonstrates the link between proliferation and

dopaminergic induction. %TH+ indicates the percentage of C17.2

cells after co-culture for 9 DIV expressing tyrosine

hydroxylase. %BrdU+ indicates the percentage of those cells

25   which incorporated BrdU.

<u>Figure 3</u> shows that VM Type 1 astrocytes induce a dopaminergic

phenotype on a *Nurr1* over-expressing neural stem cell line

(*Nurr1*-C17.2-clone 42). %TH+ indicates the percentage of clone

30   42 cells expressing tyrosine hydroxylase. (E16 VM) co-culture

of clone 42 with E16 ventral mesencephalon cells; (Tot) co-

culture with total primary cells; (Ad+) co-culture with

adherent cell fraction; (Ad-) co-culture with non-adherent

cell fraction; (T1A) co-culture with Type 1 astrocytes; (P1

35   T1A) co-culture with Postnatal day 1 Type 1 astrocytes from

the ventral mesencephalon. (Insert) insert separates clone 42

cells from P1 T1A cells in co-culture; (CTX) co-culture with
cortex, (HC) co-culture with hippocampus; (SC) co-culture with
spinal cord.

5   Figure 4 illustrates HPLC analysis of supernatants collected
from co-cultures of Type 1 astrocytes of the ventral
mesencephalon (T1A) with either KCl-depolarized *Nurr1*-C17.2-
clone42 (c42) cells, or KCl depolarized parental C17.2 (C17-2)
cells. (+SR+bFGF) co-cultures plus of bFGF and SR11237.

10

Figure 5 shows that early activity of *Nurr1* produces long-
lasting changes in gene expression in C17.2 cells. Relative
light units (RLU) indicates the expression of luciferase from
a *Nurr1* activated NBRE-luciferase reporter.

15

Detailed Description

METHODS

Nurr1-over-expressing cell lines
20   C17.2 cells were cotransfected with a CMX-Nurr1 expression
vector (Perlmann, T. et al. Genes Dev. 9, 769-782 (1995)) and
PGK-puromycin resistance plasmids (*Nurr1* clones) or a
PGK-puromycin alone (mock clones). For reporter assays, C17.2
parental cells were transfected with the *Nurr1* expression
25   vector, the reporter plasmid (NGFIB-binding response element
(NBRE) triplet upstream of TK minimal promoter fused to
firefly luciferase) and a pRSV-alkaline phosphatase plasmid at
a 2:1:2 ratio.

30   Fifty *Nurr1* transfected clones were selected for puromycin
resistance, isolated, amplified and *Nurr1* mRNA expression was
analyzed by RNase protection assay (RPA). Assays were
performed using the RPAII Ribonuclease Protection Assay Kit
(Ambion), following the manufacturers' recommendations. A 288
35   bp antisense *Nurr1* cRNA probe spanning nucleotides 1798-2086

of the mouse *Nurr1* cDNA sequence (Law, S.W. et al. Mol. Endocrinol. 6, 2129-2135 (1992)) was transcribed with T7 (from a *Nurr1* cDNA cloned into the EcoRI/XhoI site of the PBS-KS+ vector, linearised with EcoRI) and labeled with (α-32P)CTP

5   (Amersham). Protected cRNA fragments were separated on 4% PAGE under denaturing conditions. The intensity of the signal was analyzed with a phosphoimager MD storm 840 and *Nurr1* signal was standardized to the content of GAPDH in every sample.

10

Cell culture and treatments

C17.2 neural stem cells (Snyder, E. Y. et al. Cell 68, 33-51 (1992)) were maintained and passaged as previously described (Snyder, E. Y. et al. Cell 68, 33-51 (1992)). Ventral

15   mesencephala from E16 rat embryos were dissected, mechanically dissociated and plated at a final density of 1×10^5 cells/cm^2 on poly-D-lysine coated culture wells in a defined, serum-free medium (N2, consisting of a 1:1 mixture of F12 and DMEM containing insulin (10 ng/ml), transferrin (100 μg/ml),

20   putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glucose (6 mg/ml), and bovine serum albumin (1 mg/ml)). After 24 hours *in vitro*, co-cultures were initiated by directly plating $2.5-5 \times 10^4$ C17.2-derived cells into the primary cultures; all ages of cultures given use this point as 0 DIV.

25   This sequence of plating and ratio of primary/C17.2 cells resulted in the healthiest cultures, although varying the numbers of C17.2 cells over a 10-fold range did not significantly affect the proportion of TH+ cells observed. Purified Type 1 astrocytes were obtained from mixed glial

30   cultures derived from various regions of P1 rats according to a standard protocol (McCarthy, K.D. et al. J. Cell Biol. 85, 890-902 (1980)). After replating into 6- or 12-well plates, astrocytes were grown to confluency in serum-containing media and changed to N2 medium. After 3-5 DIV, co-cultures were

35   initiated in fresh N2 as described above. All factors were added once, at the initiation of co-culture (concentrations are noted in the Results and Discussion), with the exception

30

of 5-bromodeoxyuridine (BrdU), which was added 4-6 hours prior
to fixation. Cultures were maintained in a humidified 5% CO_2,
95% air incubator at 37°C and fixed after given time periods
with 4% paraformaldehyde for 45 minutes for immunocytochemical
5   analysis.

Immunocytochemical analysis and HPLC

Fixed cultures were incubated with one of the following
antibodies, diluted appropriately in phosphate-buffered saline
10   (PBS) containing 1% bovine serum albumin and 0.3% Triton-X
100: mouse anti-BrdU, 1:50 (DAKO, Denmark), mouse
anti-β-tubulin, Type III (TuJ1), 1:250 (Sigma), mouse anti-TH,
1:1000 (Incstar, USA), rabbit anti-β-galactosidase, 1:250
(Cappel, USA) rabbit anti-GFAP, 1:500 (DAKO, Denmark), rabbit
15   anti-AHD-2, 1:4000. Incubations were either carried out at 4°C
overnight, or at room temperature for 1 hour. Both processes
yielded similar results. After washing, cultures were
incubated for 1-3 hr with appropriate secondary antibodies
(biotinylated horse-anti-mouse IgG or goat-anti-rabbit IgG,
20   Vector, USA), 1:100, in the same dilution buffer.
Immunostaining was visualized with the Vector Laboratory ABC
immunoperoxidase kit, using either AEC (red) or SG (grey)
substrates. Fluorescent double-labeling of β-galactosidase
(β-gal) was performed by substituting the biotinylated
25   secondary with FITC-conjugated antibody (Vector, USA), 1:100.
Quantitative immunocytochemical data represent means and
standard errors from 100-500 cells counted in each of 3-6
separate wells from 2-4 experiments. For quantitation of TH
expression in co-cultures of primary and C-17.2-derived cells,
30   TH was visualized with the brightfield AEC substrate, while
β-gal was assessed using FITC, thus all data expressed as
"%TH+" represents the number of TH+/β-gal+ divided by total
β-gal positive cells.

35   For analysis of dopamine release, large (10 cm) co-cultures
containing approximately 1 million *Nurr1*-C17.2-c42 or C17.2
parental cells with P1 VM T1A were treated with 200 μl of 50

mM KCl in PBS/ 0.1 M sodium citrate for 5 minutes while swirling. The supernatants were immediately assayed for dopamine content using high-pressure liquid chromatography (HPLC). Samples were separated with a reverse-phase C-18

5   column, eluted with acetonitrile and detected electrochemically. Results were verified with a standard containing dopamine, DOPAC, 5-HT, and 5-HIAA.

Long-term cultures and transplantation

10 Parental C17.2 cells or C17.2-*Nurr1*-c42 cells were grown in large (10cm) insert cocultures with VM type 1 astrocytes for 8 days in the presence of bFGF (10ng/ml) and SR11237 (1μM). Cells were then trypsinized off of the insert, pelleted, and resuspended, at a concentration of 100,000 cells/μl, in their

15 own conditioned media; an aliquot of this mixture was then plated into a poly-D-lysine treated 6-well tissue culture plate containing N2 media, while the remainder was used for transplantation. Adult (25-30 grams) CD1 mice housed and treated according to the guidelines of the European Community

20 (86/609/EEC) were anaesthetized with pentobarbital (60mg/kg i.p.). 25,000 cells were stereotaxically injected into the striatum, at each of the two following coordinates (in mm): AP (bregma) = 0.56, L=1.9, DV(dura) = -2.55 and -2.75, with the incisor bar at -3. Twelve days after grafting, mice were

25 transcardially perfused with 4% paraformaldehyde. Brains were postfixed for 2 hours, embedded in 10% sucrose for more than 1 day and frozen in dry-ice cooled isopentane. 14 micron cryostat sections were processed for TH immunohistochemistry using mouse anti-TH (Incstar, Minnesota, USA) 1:1000, and

30 donkey anti-mouse rhodamine (Jackson, Pennsylvania, USA) 1:100 antibodies. TH-positive cells were photographed using a Hamamatsu camera attached to a Zeiss Axioplan 2 microscope.

RESULTS AND DISCUSSION

35

Expression of *Nurr1* above basal levels in multipotent neural stem cells

The inventors used a well characterized clone of multipotent
neural stem cells termed C17.2 (Snyder, E. Y. et al. Cell 68,
33-51 (1992)). Initially derived from developing mouse
cerebellum, C17.2 cells have been immortalized with v-myc,
5   contain a lacZ reporter, possess the ability to differentiate
into neurons, astrocytes, and oligodendrocytes *in vitro* and *in
vivo*, and upon transplantation into the developing brain,
adopt regionally-appropriate neuronal phenotypes (Snyder, E.
Y. et al. Cell 68, 33-51 (1992); Snyder, E. Y. et al. Nature
10   374, 367-370 (1995)). Moreover, the same single factors known
to direct the differentiation of primary stem cells from the
fetal and adult central nervous system (Johe, K. K. et al.
Genes Dev. 10, 3129-3140 (1996)) direct the differentiation of
C17.2 cells *in vitro*.

15

C17.2 cells were stably transfected as described below and
fifty *Nurr1* clones were analyzed for transgene expression by
RNase protection assay (RPA). Several *Nurr1* clones over-
expressed the transgene, of which the five highest expressors
20   were selected for analysis. These five selected clones
expressed much higher levels of *Nurr1* than parental C17.2
cells, or cells of the ventral mesencephalon, cerebellum or
cortex. 8 randomly chosen, mock control clones were also
selected. All *Nurr1* clones behaved similarly to the parental
25   and mock clones in serum-containing media, with no obvious
differences in growth rate or morphology.

Differentiative capacity of *Nurr1* over-expressing clones

To define the differentiative capacity and phenotypic fate of
30   *Nurr1* over-expressing clones, the inventors examined the
behavior of the clones after low density passage into
serum-free, defined media (conditions which allow for
differentiation). In this condition, the parental cell line
begins to differentiate, so that after 4-5 days *in vitro*
35   (DIV), 80-85% of the population is post-mitotic (i.e. BrdU

negative after extended pulse), while approximately 20-30% of the post-mitotic cells adopt a neuronal fate, judged by expression of β-tubulin III (TuJ1).

5   The effects of *Nurr1* on differentiative capacity, as measured by incorporation of 10μM BrdU after a 6 hour pulse in serum-free media, were varied. Although there was a trend toward increased differentiation within the *Nurr1* clones, no clear effect of the transgene on this process was seen (Fig 1A).

10   However, the effects of *Nurr1* on the fate of post-mitotic cells were clear and robust (Fig. 1B). Expression of the *Nurr1* transgene significantly increased neuronal fate in serum-free media, as judged by expression of β-tubulin III (TuJ1), to an average of 68% of the post-mitotic cells across

15   all five clones (global effect of *Nurr1* transgene vs. mock, *P < 0.0001 by 2-way ANOVA). In four of five *Nurr1* clones, the vast majority of post-mitotic cells (greater than 60%) adopted a neuronal fate, a phenomenon not seen in any of the mock clones.

20

Although *Nurr1* appears to restrict the C17.2 cell line to neuronal lineages, the neurochemical fate of these neurons was not dopaminergic as no significant tyrosine hydroxylase (TH, a marker for dopaminergic neurons) immunoreactivity was detected

25   in any of the *Nurr1* clones under these conditions.

Induction of *Nurr1* over-expressing clones to the dopaminergic fate

The inventors treated the *Nurr1* clones with a variety of

30   trophic factors, mitogens, cytokines and other agents known to be important in the proliferation (basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor, fetal calf serum), differentiation (retinoic acid, dopamine, forskolin, sonic hedgehog) and survival (glial

35   cell line-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, ciliary neurotrophic

factor) of endogenous dopaminergic neurons. None of these
factors induced TH expression in any of the clones, alone or
combined.

5   The *Nurr1* clones were co-cultured *in vitro* with primary
     cultures derived from E16 rat ventral mesencephalon, the age
     and region where endogenous dopaminergic neurons of the
     substantia nigra have just been born (Altman, J. et al.
     Neurogenesis and Neuronal Migration, in The Rat Nervous
10   System, 2nd edition (ed. Paxinos,G. Academic Press, San Diego)
     p. 1054 (1995)). Under these conditions a significant
     percentage of cells from two of the *Nurr1* lines (the clones
     with the highest proliferative capacity, clones 4 and 42)
     demonstrated measurable amounts of TH immunoreactivity (Fig.
15   1C - *$P < 0.0001$, interaction of transgene vs. clone by 2-way
     ANOVA). Little or no TH staining was seen in the C17.2 parent
     or any of the mock control lines. Phase-contrast microscopy
     of the cocultures revealed large proliferative clusters of
     Nurr1-clone-derived TH+ cells.

20

     Collectively, these observations suggested that one or more
     local factors derived from the primary cultures interacted
     directly or indirectly with *Nurr1* to induce TH expressing
     neurons. TH expression was limited to a minority
25   subpopulation within a fraction of the *Nurr1* clones.

     Treatment of co-cultures with SR11237, bFGF and EGF
     Treating co-cultures of the highest TH-expressing *Nurr1* clone
     (clone 42) and primary VM cells with combinations of the
30   previously mentioned factors was generally ineffective in
     increasing TH expression, with three important exceptions:
     the synthetic retinoid analog SR11237, bFGF, and EGF (Fig.
     2A).

35   Addition of SR11237 (to 1μM) and bFGF (to 10ng/ml) to the
     parental C17.2 cells in co-culture did not induce expression

of TH, however, each of these molecules induced TH expression in 40-60% of the *Nurr1*-C17.2 clone 42 population when added to co-cultures in serum free media. The effects of bFGF could be substituted by EGF. Moreover, the effects of SR11237 and bFGF

5 or EGF were additive at saturating doses (up to 90% LacZ+ were TH+) suggesting that they may act through distinct mechanisms.

SR11237 specifically stimulates RXR retinoid receptors (Lehmann, J.M. et al. Science 258, 1944-1946 (1992)) which

10 have been shown to heterodimerize with *Nurr1* to form transcription-initiating complexes (Perlmann, T. et al. Genes Dev. 9, 769-782 (1995)). This observation, along with observations that all-trans retinoic acid was without effect, suggest that a functional interaction between *Nurr1*, RXR

15 receptors and primary VM cells may be involved.

Retinoids and FGF have previously been shown to play a role in normal dopaminergic neuronal development (Ye, W. et al. Cell 93, 755-766 (1998); Eichele, G. Trends Genet 13, 343-345

20 (1997); Krezel, W. Science 279, 864-867 (1998)). However, although these factors increase the proportion of TH expressing cells as discussed above, the inventors have shown that the effects of these factors on *Nurr1*-C17.2-c42 (c42) seem to be modulatory. Antagonism of either, with blocking

25 antibodies or with specific RAR or RXR antagonists, did not prevent basal TH expression of c42 in co-culture (i.e. about 10%, Fig. 1C). Antagonism of sonic hedgehog using blocking antibodies was also ineffective in preventing basal TH expression. Thus, the only obligate requirement for the

30 induction of a dopaminergic phenotype in c42 seems to be exposure to signals derived from primary ventral mesencephalic cells.

Pretreatment of clones with bFGF and EGF
35 bFGF and EGF are both directly mitogenic to the C17.2 cells (Kitchens, D.L. et al. J. Neurobiol 25 (7), 797-807 (1994)), but it was found that after extended treatment with either

factor in serum-free medium (SFM), the baseline proliferation of most clonal lines remained unexpectedly elevated after passage into SFM alone. Moreover, it was surprising that *Nurr1* clones with the highest amount of residual proliferation

5   in serum free medium (clones 4 and 42), demonstrated significant TH expression in co-culture.

The inventors reasoned that increasing the proliferation of other *Nurr1* clones should increase the number of TH expressing

10 neurons. *Nurr1* clones were pretreated with bFGF for 5 DIV prior to splitting and replating into primary ventral mesencephalic cultures. Sister cultures were assayed for both proliferation after pretreatment and TH expression after co-culture for 9 DIV. This procedure enabled selective

15 examination of the effects of bFGF on the *Nurr1* clones and eliminated the mitogenic effects of bFGF on primary cells as well as its indirect trophic effect on primary dopaminergic neurons.

20 BrdU labeling of bFGF-pretreated *Nurr1* clones was increased at 24 hours after passage of cells into co-culture, as compared with cells directly split out of serum-containing medium (rightward shift of individual clones on X-axis, Fig. 2B). — Concomitant with this increase in proliferation, proportional

25 increases in the percentage of TH-positive cells were observed in all of the *Nurr1* clones (upward shift of clones on Y-axis, Fig. 2B), reaching as high as 45% TH+ (clone 42). In fact, regression analysis indicates a significant linear relationship ($r^2=0.890$ by regression) between the proliferative

30 capacity of the *Nurr1* over-expressing clones at the initiation of co-culture and the propensity to express TH after differentiation within, as well as between, individual clones after 9 DIV.

35 The above results therefore suggest that *Nurr1* over-expressing clones should preferably be mitotic when first brought into

contact with primary VM cells, or with one or more factors supplied by or derived from primary VM cells, i.e. that an important influence on differentiation to a dopaminergic fate in *Nurr1* clones is their proliferative capacity.

5

Further support is provided by the observation that, at the time when most TH-expressing c42 cells begin appearing in co-culture (6 DIV), nearly all of them are still mitotic (BrdU positive after acute pulsing at 6 DIV. However, after similar

10   treatment of co-cultures at 9 DIV, the vast majority of TH+ cells are BrdU negative, suggesting withdrawal from the cell cycle following induction of measurable TH expression.

Previous studies examining the adoption of phenotypic fate

15   during cerebral cortical (McConnell, S. K. et al. Science 254, 282-285 (1991)) and spinal cord (Ericson, J. et al. Cell 87, 661-673 (1996)) development have demonstrated that exposure of non-committed neuroblasts to spatially-restricted, local factors induces specific phenotypes within these populations,

20   but this induction is contingent upon continuous exposure up to and including the terminal S-phase of the neuroblast. A similar mechanism may underlie the present observations.

<u>Analysis of the inductive signal from primary ventral</u>

25   <u>mesencephalic cells</u>
Primary ventral mesencephalic cultures typically contain a mixture of dopaminergic and other neurons, astrocytes, oligodendrocytes, as well as assorted non-neural elements such as microglia, endothelial cells, and fibroblasts. To identify

30   the source of the TH-promoting activity, the inventors performed a crude separation of the primary cells based on adhesion; the rapidly adherent population was enriched for glial and non-neural elements, while the non-adherent population consisted mainly of neurons, oligodendrocyte

35   precursors, and a few astrocytes.

Co-culture of these fractions with c42 demonstrated that the

38

majority of TH-inducing activity was contained within the rapidly adherent population of E16 ventral mesencephalon cells (Fig. 3). The inventors then prepared purified cultures of Type 1 astrocytes from E16 ventral mesencephalon and
5   demonstrated that T1A were the source of TH-inducing activity: in the absence of any added factor, the number dopaminergic cells derived from the *Nurr1*-c42 line dramatically increased. Furthermore, this activity was not restricted to early development, as astrocytes isolated from the VM of newborn
10   rats induced equivalent numbers of TH positive c42 cells (about 70%) (Fig. 3).

Nurr1-C17.2-c42 cells were treated with Type 1 astrocyte conditioned media or membrane fragments, respectively.
15   However, neither of these treatments induced a significant increase in the number of TH expressing cells, suggesting that this inducing activity was highly labile. The inventors co-cultured astrocytes and *Nurr1*-C17.2-c42 cells, but spatially separated the two populations (by about 1mm) via a microporous
20   insert (0.4μm porous membrane). The insert allowed free passage of macromolecules but prevented contact between the two populations. In this environment, TH expression was induced in *Nurr1*-C17.2-c42 cells at a level equivalent to direct co-culture (Fig. 3). These results provide indication
25   that ventral mesencephalic Type 1 astrocytes secrete a highly labile diffusible factor which interacts with *Nurr1* over-expressing lines to induce TH expression.

<u>Inductive activity of T1A from other brain regions</u>
30   The inventors then examined whether the inductive activity is restricted to Type 1 astrocytes from the ventral mesencephalon. T1A were isolated from several brain regions which contain populations of *Nurr1*-expressing cells during development (Zetterström, R. H. et al. Mol. Brain Res. 41,
35   111-120 (1996)). No increase in the number of TH immunoreactive cells was observed in c42, compared to parental

C17.2 cells, when co-cultured with T1A from other brain
regions including cerebrocortex, hippocampus, or spinal cord
(Fig. 3). This indicates that a putative TH-inducing factor
is specifically and selectively produced by ventral

5   mesencephalic Type 1 astrocytes.

However, c42 cells were not unaffected by astrocytes from
these regions. Cells of this line developed distinct,
neuronal-like morphologies unique to each region. Under the

10   same conditions however, the C17.2 parental line tended to
display a uniform mixture of polygonal, bi- and tripolar
morphologies. Collectively, these data suggest that
astrocytes from distinct brain regions secrete unique, or
unique combinations of, factors which interact with

15   *Nurr1*-expressing cells to produce specific, and perhaps

regionally appropriate, neuronal phenotypes. Confirmation is
obtained by identifying the neuronal phenotypes which are
produced in the co-cultures. Specific neuronal markers are
used, such as NPY, substance P, Ach, and Islet 1. Further

20   support is obtained by grafting C17.2 cells expressing *Nurr1*

above basal levels intracerebro-ventricularly at early
embryonic stages to allow for their integration. The neuronal
phenotypes originated by these cells are determined by lacZ
expression and immunohistochemistry against a specific

25   neuronal marker.

The mechanism of dopaminergic induction
The inventors compared *Nurr1* activity in C17.2 cells shortly
after transfection with *Nurr1* activity in established C17.2-

30   *Nurr1* clones. They found that significant *Nurr1*

transactivational activity was detected 36 hours after
cotransfection of the *Nurr1* expression vector and a *Nurr1*-
responsive NBRE-luciferase reporter into C17.2 cells. No
significant increase in basal *Nurr1* transactivational activity

35   was observed after transfection of the reporter into stable,
proliferating C17.2-*Nurr1* clones (grown in serum) (Figure 5),

40

or after transfection into differentiated clones obtained
after passage into serum free medium.

These data indicate that *Nurr1* is not active during the

5   neuronal differentiation of stable C17.2-*Nurr1* clones, and
suggest that earlier transient high levels of *Nurr1* activity
may have conferred upon the *Nurr1* clones long-lasting
competence to be dopaminergic.

10   Changes in gene expression in *Nurr1* clones
The inventors examined changes in gene expression in *Nurr1*
clones. GFR α1 and GFR α2 mRNA levels in mock and *Nurr1* clones
were examined using RNase protection assays (GDNF familiy
receptor α1; GDNF refers to Glial cell line-Derived

15   Neurotropic Factor). C17.2 and all the mock clones examined
expressed high levels of GFR α1 mRNA and very low levels of
the related receptor GFR α2. In contrast, all *Nurr1* clones
demonstrated the inverse profile, i.e. very low GFR α1 and
very high GFR α2 mRNA levels, suggesting that in C17.2 cells ,

20   *Nurr1* does not directly induce the TH+ phenotype, but rather
acts indirectly, by producing stable up- or down-regulation of
relevant proteins. In such a way, *Nurr1* may bestow
multipotent cells with the competence to respond to specific
factors, including those derived from ventral mesencephalic

25   astrocytes.

The effects of *Nurr1* activity on the pattern of gene
expression is further investigated by analysing the expression
of genes important for the specification of midbrain neurons

30   and ventral phenotypes (e.g. Shh, Patch, Smo, Gli genes, Hes,
FGF8, Ptx3, Pax genes, Engrailed 1 and 2) and genes important
for neurogenesis and differentiation (e.g. Noggin, Chordin,
Follistatin, Mash1, Math1, NeuroD, Neurogenin1-3, Notch1-3,
Delta, Serrate and Myt1).

35
     The dopaminergic phenotype

The gain of TH expression represented the adoption of a
legitimate dopaminergic phenotype within the *Nurr*1-C17.2-c42
line. By convention, the ability to release dopamine in
response to membrane depolarization is the vital criterion for
5   designation of neurochemical phenotype as dopaminergic.
Co-cultures of parental or c42 lines and VM astrocytes were
thus treated acutely with 50mM KCl and the supernatants were
assayed for monoamine content with HPLC. Significant release
of dopamine and its metabolite DOPAC was detected in
10   co-cultures containing c42, with increased release detected in
co-cultures treated with factors enhancing dopaminergic
differentiation, e.g. SR11237 and bFGF, thereby correlating
dopamine release to the number of TH expressing cells (Fig.
4). No dopamine release was observed in the parental line.
15   Thus, TH expressing *Nurr*1-C17.2-c42 cells may be considered
truly dopaminergic.

The TH expressing *Nurr*1-C17.2-c42 line was of a ventral
mesencephalic dopaminergic type. *Nurr*1-C17.2-c42 cells in
20   co-culture acquired immunoreactivity for aldehyde
dehydrogenase-2 (AHD-2; Fig. 4), an enzyme selectively
expressed in developing dopaminergic precursors within the
ventral mesencephalon (McCaffery, P. et al. Proc. Natl. Acad.
Sci. USA 91, 7772-7776 (1994)). They also expressed c-ret
25   mRNA, the signaling receptor for GDNF (Trupp, M. et al. Nature
381, 785-789 (1996); Jing, S. Q. et al. Cell 85, 1113-1124
(1996)) which is present in dopaminergic neurons (Trupp, M. et
al. Nature 381, 785-789 (1996)). Furthermore, TH positive c42
cells demonstrated similar responses to factors with known
30   neurotrophic effects on dopaminergic progenitors (Studer, L.
et al. Nature Neurosci. 1, 290-295 (1998)) and primary ventral
mesencephalic dopaminergic neurons *in vitro* (Hyman, C. et al.
J. Neurosci. 14 ,335-347 (1994); Lin, L. H. et al. Science
260, 1130-1173 (1993)) (Fig. 4). Co-cultures were first
35   treated with SR11237 and bFGF to induce a dopaminergic
phenotype, after which the media was changed to N2 with or
without various growth factors. At 9 DIV bFGF and

42

neurotrophin-3 (NT-3 30ng/ml) dramatically increased the number of TH+ cells in the culture, compared to the N2 control condition; Brain Derived Neurotrophic Factor (BDNF, 30ng/ml), Ciliary Neurotrophic Factor (CNTF, 10ng/ml) and Glial cell

5   line-Derived Neurotrophic Factor (GDNF, 10ng/ml) induced neuritogenesis and/or hypertrophy on *Nurr1*-c42-derived dopaminergic neurons. Thus the behavior of TH expressing c42 cells parallels that of endogenous neurons of the substantia nigra, so these cells are ventral mesencephalic-like

10   dopaminergic neurons.

Stability of induced dopaminergic neurons
The inventors assessed the long term stability of the induced dopaminergic phenotype *in vitro* and *in vivo*, particularly

15   after removal of astrocyte-derived inductive factors. c42 cells were co-cultured in inserts with VM type 1 astrocytes for 8 days, removed from the inserts and replated into defined N2 media without additional factors for 14 days. Although some cellular attrition was observed in the two weeks after

20   removal from co-culture, a significant number of cells displayed a highly mature dopaminergic phenotype, including long elaborate processes, hypertrophic cell bodies and intense levels of TH-immunoreactivity.

25   c42 cells from parallel co-cultures were also surgically injected into adult mouse corpus striatum and allowed to mature for 12 days, in the absence of any additional tropic factors or supportive cells (i.e. astrocytes, oligodendrocytes, or other neurons). Although cells were lost

30   in this condition, a small but significant number of c42 derived dopaminergic cells displayed a high level of differentiation and apparent integration into the host tissue. No TH+ cells derived from the c17.2 parental line were found in either of the long term *in vitro* or *in vivo* experiments.

35   Thus the observation that some c42 cells maintained or even increased TH expression after removal from Type 1-derived factors shows that, after dopaminergic induction, their

phenotype is stable. Since only a limited number of surviving TH+ cells could be detected, exogenously applied tropic factors or supporting cells are required for long-term survival.

5

Treatment of neurodegenerative disease

Confirmation of the ability of c17.2 derived neurons to treat neurodegenerative disease is obtained using an *in vivo* model of Parkinson's disease. The false neurotransmitters 6-

10 hydroxydopamine (6-OHDA)or MPTP are specifically taken up by neurons and lead to oxidative stress and loss of dopaminergic and noradrenergic neurons.

Nurr1-c17.2 cells that have been differentiated into

15 dopaminergic neurons *in vitro* are surgically implanted into either the substantia nigra or the striatum of 6-OHDA treated mice. The ability of these cells to integrate and fully differentiate is morphologically evaluated by β-gal staining, TH-immunohistochemistry and in situ hybridisation for dopamine

20 transporter and dopamine receptors.

The ability of undifferentiated *Nurr1*-c17.2 cells to spontaneously differentiate *in vivo* toward the dopaminergic phenotype is assessed by intrastriatal or intranigral grafting

25 of such cells into 6-OHDA treated animals. The dopaminerigc phenotype is detected as described above.

The ability of *Nurr1*-c17.2 cells grafted into the striatum or substantia nigra to rescue motor asymmetries induced by

30 unilateral 6-OHDA treatment is confirmed by assessment of circling behaviour in apomorphine and amphetamine tests (Schwarting, R. K., et al., (1996) Progress in Neurobiology, *50(2-3)*, 275-331).

35 *SUMMARY*

In conclusion, the results presented herein demonstrate that

expression of *Nurr1* above basal levels in neural stem or progenitor cells induces a neuronal fate. Moreover, Type 1 astrocytes of the ventral mesencephalon, but not from other brain regions, release one or more soluble factors which

5   induce neural stem or progenitor cells originally obtained from a non-dopaminergic brain region and which express *Nurr1* above basal levels, to develop into dopaminergic neurons of the ventral mesencephalon. Thus dopaminergic neurons can be generated from neural stem or progenitor cells through a

10   process including expression of *Nurr1* above basal levels in the cells and contact of the cells with one or more factors supplied by or derived from type 1 astrocytes of the ventral mesencephalon. In addition, the results suggest that primary astrocytes may be the source of signals required for the

15   induction of regionally appropriate neuronal phenotypes in multiple brain structures.

The methods described herein, which take advantage of the multipotential capacity of neural stem or progenitor cells,

20   selector genes such as *Nurr1*, and primary astrocytes, provide for the production of neurons of a desired neurochemical phenotype as a source material for neuronal transplantation in the treatment of neurodegenerative diseases. The induction of midbrain dopaminergic neurons may be used in a cell

25   replacement strategy to treat Parkinson's disease.